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Byproducts of protein, lipid and DNA oxidative damage and antioxidant enzyme activities in seizure

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ABSTRACT

Purpose: To get more insight into molecular mechanisms underlying oxidative stress and its role in different types of seizure, in this study, oxidative byproducts of proteins, lipids and DNA, as well as, antioxidant enzyme activities were studied in adult patients with epilepsy.
Methods: Study was performed in 60 patients with epilepsy and in 25 healthy controls. Plasma protein reactive carbonyl derivatives (RCD) and protein thiol groups (P-SH), byproducts of oxidative protein damage, as well as antioxidant enzyme activities, superoxide dismutase (SOD) and glutathione peroxidase (GPX) were studied spectrophotometrically. Urinary 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), representative byproducts of lipid and DNA oxidative damage, respectively, were determined by enzyme immunoassay.
Results: RCD levels were significantly increased ($p = 0.001$), while P-SH content was decreased in patients with first seizure ($p = 0.052$) compared to controls, independently of the seizure type. Urinary 8-epi-$PGF_{2\alpha}$ and 8-OHdG were significantly increased in patients with epilepsy ($p = 0.001$ and $p = 0.001$). Rise in 8-epi-$PGF_{2\alpha}$ was more pronounced in patients with generalized tonic–clonic seizure (GTCS) compared to those with partial seizure (PS). Both SOD and GPX activity were significantly increased in epileptic patients compared to controls ($p = 0.001$ and $p = 0.001$), but only SOD activity was significantly higher in patients with GTCS than in those with PS.
Conclusions: Data on enhanced protein, lipid and DNA oxidation, together with upregulated antioxidant enzyme activities, confirm the existence of systemic oxidative stress in patients with epilepsy. It might be speculated that post-translational modification to existing functional proteins, particularly alterations to ion channels, might be at least partially responsible for acute early changes in neuronal networks.

1. Introduction

In the last decade, important advances have been made in the diagnosis and treatment of seizure disorders. However, our understanding of the cellular and molecular mechanisms underlying epileptogenesis, is still incomplete. Among various factors supposed to play role in epilepsy, the role of oxidative stress and reactive oxygen species (ROS) in seizure disorders has emerged recently.[1–3] Oxidative stress, recognized as one of the predisposing factors in various neurological disorders, describes the pathologic condition in which the balance of oxidant generation and detoxification is tipped toward a prooxidant state, antioxidant defenses are overwhelmed and reactive species accumulate.[4] If left unopposed, oxidative stress results in the accumulation of dysfunctional proteins, lipid peroxidation products and damaged nuclear or mitochondrial DNA.

The previous findings on increased oxidative stress in adult epileptic patients are based on increased plasma malodialdehyde (MDA) levels, a biomarker of lipid peroxidation.[2,5] Accumulation of ROS byproducts from oxidized genomic DNA has also been demonstrated in individuals with epilepsy.[6,7] However, the data on isoprostane excretion, the most reliable *in vivo* marker of lipid peroxidation and oxidative stress are lacking. In addition, the action of free radicals, both intracellular and extracellular, is also directed to proteins as one of the major target. Moreover, oxidative damage to proteins is a major mechanism underlying neurodegeneration, which occurs through a variety of pathways including carbonylation, oxidation of critical sulfhydryl groups and nitrosylation.[8] Although epilepsy is not classified as a neurodegenerative disease *per se*, epileptic activity can lead to the cell loss and

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neurodegeneration, which contributes to disease progression.[9]. Still, there are no reliable data on markers of protein oxidation, such as protein reactive carbonyl derivatives (RCD) and protein thiol groups (P-SH) in these patients. In attempt to elucidate whether changed antioxidant enzyme activities contribute to oxidative stress in epileptic patients, glutathione peroxidase (GPX) and superoxide dismutase (SOD), have been investigated much more, but conflicting results have been obtained.[1,3,10]

Despite many evidences which suggest that epileptic activity is a state of oxidative stress, the mechanisms by which free radicals are contributing to seizure disorders remain still elusive. Namely, it has been reported that increased generation of free radicals or reduced activity of antioxidative defense mechanisms can cause some types of epilepsy and in addition, increases the risk of seizure recurrence.[2,11,12] On the other hand, studies in animal models showed the seizures *per se* may result in free radical production and oxidative damage to lipids and DNA.[13,14]

To get more insight into molecular mechanisms underlying oxidative stress and its role in different types of seizure, a systematic investigation of various components of antioxidant system together with markers of oxidative protein, lipid and DNA damage in patients with epilepsy is necessary.

In this study, oxidative byproducts of proteins (RCD and SH-groups), lipids (8-epi-prostaglandin $F_{2\alpha}$) and DNA (8-hydroxy-2′-deoxyguanosine), as well as, antioxidant enzyme activities (SOD and GPX) were studied in adult patients with epilepsy.

2. Materials and methods

2.1. Selection of study participants

We enrolled 60 patients with first unprovoked seizure. The initial diagnostic process included physical examination, electroencephalography and neuroimaging findings (CT scan and MRI), performed in all 60 patients, within first 24 h after seizure. The subjects were 29 (48%) women and 31 (52%) men, aged between 20 and 46 years. Diagnosis of epilepsy was confirmed and limited to partial cryptogenic or generalized idiopathic seizures, according to the ILAE classification.[15] Out of the total number of patients, partial seizure (motor or complex) occurred in 27 patients (45%), while 33 patients (55%) had primary generalized tonic–clonic seizure. Duration of partial seizures was from 15 s to 1 min and from 1 min to 2 min in generalized tonic–clonic seizures. None of the patients had series of seizures or status epilepticus. Exclusion criteria for the study group were abnormal neurological examination, abnormal cerebral CT scan and MRI, psychiatric or progressive neurologic disorders, thyroid diseases and other endocrinopathies, liver, heart or kidney diseases, or the diseases that could influence the level of oxidative stress, such as diabetes mellitus, arterial hypertension and malignancies. In addition, smokers and alcohol or narcotic abusers were excluded. The control group consisted of 20 non-epileptic, otherwise healthy subjects, matched for sex and age. None of the patients or controls received any antiepileptic drugs or antioxidant supplementation. All subjects gave written informed consent to participate in the study. The ethics committee of Faculty of Medicine, University of Belgrade approved the use of human tissue for research.

2.2. Plasma and urine sampling and analytical procedure

Peripheral venous blood (5 ml) for analysis was collected in tubes over trace-element free heparin immediately after admission to Emergency Unit. Plasma was separated at 3000 rpm at 4 °C during 15 min. The supernatant was collected, aliquoted and stored at −80 °C for enzyme measurement. Determination of carbonyl and protein thiol groups was performed immediately after blood collection and plasma separation. Presence of haemolysis was followed by measurement of plasma haemoglobin and all patients with haemoglobin concentration >50 mg/L were excluded from the study. Routine biochemical profiles were measured using an autoanalyser.

For 8-epi-prostaglandin $F_{2\alpha}$ and 8-hydroxy-2′-deoxyguanosine measurement, urine was collected from all patients and controls. Samples of 20 ml were centrifuged, aliquoted and stored at −80 °C. Butylated hydroxytoluene was added to prevent oxidation during processing.

2.3. Laboratory methods

2.3.1. Determination of protein reactive carbonyl derivatives in plasma

Plasma RCD were determined using the method of Levine et al.[16] The protein oxidation level was monitored by determination of a classic carbonyl reagent, 2,4-dinitrophenylhydrazine (DNPH) activity of proteins. Spectrophotometric measurement of plasma RCD was performed and expressed as μmol/g proteins. Protein concentration was determined by the method of Lowry et al.[17]

2.3.2. Determination of plasma thiol groups

The amount of plasma thiol (P-SH) groups was determined according to the method of Jocelyn[18] and expressed as μmol/g of proteins (μmol/g prot).

2.3.3. Measurement of urinary 8-epi-prostaglandin $F_{2\alpha}$

8-Epi-$PGF_{2\alpha}$ was determined by enzyme immunoassay (Bioxytech Urinary 8-Epi-prostaglandin $F_{2\alpha}$ kit; Oxis Research, Portland, OH, USA). The results were standardized against urinary creatinine concentrations and expressed in ng/mg creatinine.

2.3.4. Measurement of urinary 8-hydroxy-2′-deoxyguanosine

8-OHdG was determined by enzyme immunoassay (Bioxytech 8-OHdG-EIA kit; Oxis Research, Portland, OH, USA). The results were standardized against urinary creatinine concentrations and expressed in ng/mg creatinine.

2.3.5. Enzyme assays

Cu, Zn SOD activity in the plasma was measured by the method of Misra and Fridovich,[19] based on the ability of SOD to inhibit autooxidation of epinephrine at alkaline pH (pH 10.2). SOD activity was determined using the calibrating curve generated by the use of standard solutions of purified SOD.

GPX activity was determined by the coupled assay procedure of Gunzler et al.[20] One unit of enzyme activity is reported as μmol NADPH oxidized per minute, assuming 6.22×10^3/L/mol/cm to be the molar absorbancy of NADPH at 340 nm.

2.4. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 10.0; SPSS Inc, Chicago, IL, USA) and Microcal Origin software (version 5.0). For each variable, values were expressed as median and confidence interval. The differences between groups were tested using non-parametric, Mann–Whitney test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Byproducts of oxidative protein damage in plasma

Data on RCD levels and P-SH content in plasma of controls and patients with first seizure are presented in Table 1. RCD levels were

Table 1
Byproducts of oxidative protein, lipid and DNA damage in controls and in patients with first seizure.

	Controls ($n=20$)	Patients with first seizure ($n=60$)	Type of seizure	
			PS ($n=27$)	GTCS ($n=33$)
RCD (μmol/g protein)	0.505[a] (0.443–0.571)	0.754 (0.664–0.844)	0.704 (0.622–0.89)	0.759 (0.746–0.994)
p		0.001	0.007	0.001
P-SH/P (μmol/g proteins)	7.07 (6.51–7.47)	6.01 (5.98–6.5)	6.14 (6.04–6.8)	6.05 (5.81–6.64)
p		NS	NS	NS
8-Epi-$PGF_{2\alpha}$ (ng/mg creatinine)	0.60 (0.55–0.66)	1.39 (1.29–1.97)	0.93 (0.81–1.57)	1.76[b] (1.60–2.37)
p		0.001	0.001	0.001
8-OHdG (ng/mg creatinine)	6.71 (4.33–9.09)	19.5 (15.2–28.2)	14.56 (7.82–30.62)	20.82 (16.6–31.0)
p		0.001	0.001	0.001

RCD: protein reactive carbonyl derivatives; P-SH/P: protein thiol groups per protein concentration; 8-Epi-$PGF_{2\alpha}$: 8-epi-prostaglandin $F_{2\alpha}$; 8-OHdG: 8-hydroxy-2′-deoxyguanosine; PS: partial seizure; GTCS: generalized tonic–clonic seizure. *p*: significance when compared to controls; NS: no statistical significance.
[a] Data are expressed as median and confidence interval.
[b] Statistically significant when compared to partial seizure.

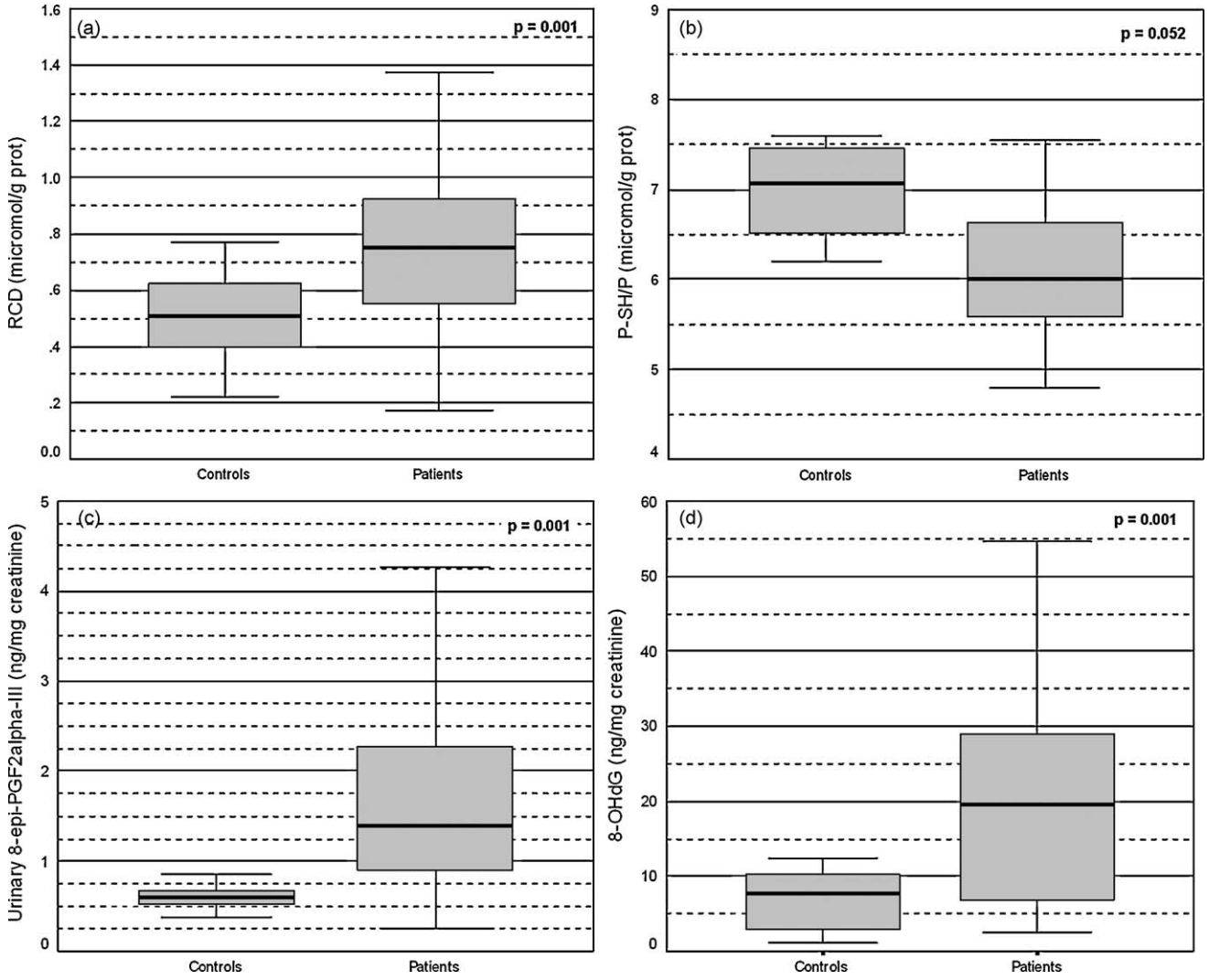

Fig. 1. Oxidative byproducts of proteins, lipids and DNA in patients with epilepsy. (a) Protein reactive carbonyl derivatives (RCD) in control group and in patients with first seizure; (b) protein thiol groups (P-SH/P) in control group and in patients with first seizure; (c) urinary 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) in controls and in patients with first seizure and (d) urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) in controls and in patients with first seizure.

significantly increased in patients with epilepsy ($p=0.001$) (Fig. 1a), independently of the type of seizure, in comparison to healthy controls. Plasma protein thiol groups content was lower in patients with first seizure, also irrespectively of the type of seizure, when compared with controls (Fig. 1b). However, this decrease in P-SH content was not significant.

3.2. Byproducts of oxidative lipid damage

The extent of lipid oxidative damage was determined by measurement of urinary 8-epi-$PGF_{2\alpha}$, a group of bioactive prostaglandin F_2-like compounds that are generated by oxidatively catalysed reaction of arachidonic acid. Data on urinary 8-epi-$PGF_{2\alpha}$

Table 2
Antioxidant enzyme activities in plasma of patients with first seizure.

	Controls ($n=20$)	Patients with first seizure ($n=60$)	Type of seizure	
			PS ($n=27$)	GTCS ($n=33$)
SOD ($U\times10^3$/L)	1.21[a] (0.96–1.42)	2.49 (2.02–2.55)	2.79 (2.39–2.91)	3.15[b] (2.82–3.49)
p		0.001	0.001	0.001
GPX (U/L)	298 (285–317)	556 (530–566)	590 (569–629)	615 (586–642)
p		0.001	0.001	0.001

SOD: superoxide dismutase; GPX: glutathione peroxidase; PS: partial seizure; GTCS: generalized tonic–clonic seizure. p: significance when compared to controls.
[a] Data are expressed as median and confidence interval.
[b] Statistically significant when compared to partial seizure.

excretion in controls and patients with first seizure are presented in Table 1. As shown, urinary 8-epi-$PGF_{2\alpha}$ excretion was significantly, more than 2-fold, increased in patients with seizure ($p = 0.001$) (Fig. 1c). Furthermore, urinary 8-epi-$PGF_{2\alpha}$ excretion was significantly higher in patients with the first generalized tonic–clonic seizure in comparison to those with partial seizure ($p = 0.001$).

3.3. Byproducts of oxidative damage to DNA

As a marker of oxidative damage to DNA, urinary excretion of 8-OHdG, a product of oxidatively modified DNA base guanine, was determined in patients with first seizure (Table 1). Urinary excretion of 8-OHdG was significantly increased ($p = 0.001$) in epileptic patients when compared to controls (Fig. 1d). Although 8-OHdG urinary excretion was higher in patients with generalized tonic–clonic seizure than in patients with partial seizure, this difference was not significant.

3.4. Plasma antioxidant enzyme activities

Table 2 summarizes the data on antioxidant enzyme activities in controls and patients with epilepsy. SOD activity was significantly increased in patients with epilepsy in comparison to controls ($p = 0.001$), especially in a group of patients with generalized tonic–clonic seizure, which further significantly differed from patients with partial seizure ($p = 0.001$). GPX activity was also significantly increased in patients with first seizure, when compared to controls ($p = 0.001$), but no significant difference was observed in different types of seizure.

4. Discussion

Our study has shown that patients with first epileptic seizure exhibit increased protein damage, based on the increased plasma RCD content and decreased levels of protein thiol groups. Oxidative modifications of proteins are accompanied by significant increase in 8-epi-$PGF_{2\alpha}$ and 8-OHdG urinary excretion, indicating increased lipid and DNA oxidative damage. Furthermore, activities of SOD and GPX in plasma of these patients are upregulated. The changes in oxidative stress parameters were more pronounced in patients with generalized tonic–clonic seizures compared to those with partial seizures, but observed differences reached statistical significance only for urinary 8-epi-$PGF_{2\alpha}$ excretion and plasma SOD activity.

Oxidative stress and generation of reactive oxygen species (ROS) are strongly implicated in a number of neurological disorders, including seizure disorders.[3] Namely, oxidative reactions occurring in mitochondria produce oxygen radicals physiologically in body tissues, as well as, in nervous system cells. If a strong antioxidant defense system is present in the cells, they will be protected from the damaging effects of ROS.[3,21] Oxidative damage to proteins is one of the major mechanisms underlying neuronal cell damage.[8] The presence of free radical-initiated reactions of side chains of amino-acid residues is indicated by DNPH-reactive carbonyl group of proteins.[22] In our study, significant changes in the DNPH activity of plasma proteins have been shown, to our knowledge, for the first time in adult patients after the first seizure. Besides, we demonstrated that the concentration of protein sulfhydryl groups in plasma, which are important chain breaking antioxidant,[23] is reduced in patients with first seizure. Decreased P-SH levels may be a consequence of enhanced free radicals production in seizure, since sulfhydryl groups of plasma proteins have been suggested to be a "sacrificial" antioxidant in plasma and extravascular spaces.[23] Until now, oxidative modifications of proteins in epilepsy have only been studied in rodent model of experimental epilepsy, by measurement of thiol redox state in mouse striatum.[24] Present results on increased carbonyl content and enhanced thiol oxidation, appear to prove the first demonstration that increased oxidative damage of plasma proteins is present in patients with first seizure. The consequences of such oxidative protein damage in epilepsy may be modified membrane and cellular function depending on the nature of the vulnerable protein component and the attacking radical species. It might be speculated that post-translational modification to existing functional proteins, particularly alterations to ion channels, might be at least partially responsible for acute early changes in neuronal networks. In addition, the neuronal network could be previously altered, for still unknown reasons, and more prone to the oxidative damage, realized after the first seizure.

Apart from protein oxidative damage, lipid peroxidation is one of the most biologically important free radicals reaction.[25] If unopposed with an effective local antioxidative defense system, peroxidative injury to plasma phospholipids may lead to the severe cell damage. The high rate of oxidative metabolism, coupled with the low antioxidant defenses and the richness in polyunsaturated fatty acids, makes the brain highly vulnerable to free radical damage.[26] The increased susceptibility of the brain to oxidative damage highlights the importance of understanding the role of oxidative stress in the pathophysiology of seizures. So far, malodialdehyde (MDA), one of the important aldehydes resulting from membrane lipid peroxidation products, or peroxidated lipids (LPO) were used as markers of lipid oxidative damage, both as measure of lipid peroxidation in brain tissue in ferric chloride and kainate-induced epilepsy in rats[27,28] and in clinical settings.[2,5,29] The 8-epi-prostaglandin $F_{2\alpha}$ (8-Epi-$PGF_{2\alpha}$), a reliable marker of free radical-induced lipid peroxidation has only been studied in animal models of epilepsy. Namely, Patel et al.[30] examined the occurrence of oxidant injury following kainate-induced seizure activity by measuring hippocampal F_2-isoprostanes. Our data on increased levels of urinary 8-Epi-$PGF_{2\alpha}$ in patients with first seizure, especially in those with generalized tonic–clonic seizures, are in agreement with findings of Patel et al.[30] who demonstrated a marked subregion-specific increase in F_2-isoprostanes following kainate administration. These data suggest that oxidative lipid damage results from seizure activity and may play an important role in seizure-induced death of vulnerable neurons.

DNA is also a major target of constant oxidative damage from endogenous oxidants.[31] Although numerous defense systems protect cellular macromolecules against oxidation, there is a high rate of damage to DNA. Currently, 8-hydroxydeoxyguanosine, a product of oxidatively modified DNA base guanine, is being used as a sensitive marker for oxidative DNA damage.[32] Namely, 8-OHdG is produced and accumulates at the area of injury, it is secreted into the bloodstream and is finally excreted in the urine.[6] While measurement of 8-OHdG in cerebrospinal fluid (CSF) is expected to be a more sensitive and immediate marker of CNS oxidative stress than its urinary levels, the collection of CSF is invasive and could be more stressfull for patients. In addition, urine tends to have more concentrated levels of 8-OHdG than CSF. Recently, Fukuda et al.[7] have demonstrated a statistically significant correlation between urinary and CSF 8-OHdG levels. In accordance to these results, our data on increased levels of 8-OHdG in patients with first seizure indicate that oxidative stress involves cascade of molecular events in which all major biological macromolecules are affected.

The question that arises is whether epilepsy or oxidative stress is the primary event and how the antioxidant enzymes are involved. Extracellular SOD represents a major defense system against superoxide, being also a target for oxidative damage.[33] Hurd et al.[34] have reported an increase in serum extracellular SOD activity in the progressive myoclonus epilepsies. Similar findings were reported by Ben-Menachem et al.[35] who found increased plasma SOD activity, but decreased erythrocyte SOD activity in patients with progressive myoclonus epilepsies. Our results on significant increase in plasma SOD activity in patients with first unprovoked seizure, especially in those with generalized tonic–clonic seizures, are in agreement with these finding. Other studies on SOD activity have mainly been performed in children with epilepsy treated by different AEDs,[1,10,36] but they reported no significant difference in SOD activity between children with epilepsy and healthy controls.

The data on another major antioxidant enzyme, GPX, seem to be conflicting too.[1,3,35] Extracellular GPX is presumed to work as part of a traditional glutathione cycle.[37] The tripeptide glutathione is involved in the disposal of peroxides by brain cells and in the protection against reactive oxygen species, while its content in brain cells depends strongly on the availability of precursors for glutathione.[38] *In vitro* extracellular GPX reduces organic hydroperoxides, phospholipid hydroperoxides and hydrogen peroxide.[39] Studies in mice that overexpress extracellular GPX suggest a protective extracellular antioxidant activity for extracellular GPX.[40] The pattern of changes observed in our study for plasma GPX activity in patients with first seizure resembles that of SOD. Namely, we have shown a significant increase in plasma GPX activity and these data are in agreement with findings of Cengiz et al.[1] who suggested that GPX upregulation might be a consequence of induced GPX synthesis in a liver as a compensatory mechanism for decreased glutathione levels detected in the same group of patients.

Upregulation of plasma SOD and GPX activities in patients with epilepsy most probably represent an adaptive phenomenon to increased free radical production in seizure. Despite of high SOD and GPX activities, accumulated RCD, lipid peroxyl radicals and their byproducts, such as 8-Epi-$PGF_{2\alpha}$, as well as, 8-OHdG overwhelm antioxidant capacity.

5. Conclusion

In conclusion, data on enhanced protein, lipid and DNA oxidation, together with upregulated antioxidant enzyme activities, confirm the existence of systemic oxidative stress in patients with first seizure. However, its actual significance in complex cascade of molecular, cellular and neuronal network alterations must be taken with some caution. Although our finding resemble to those in neuronal tissue obtained from animal models, plasma and urine constituents do not necessarily reflect damage and are not necessarily formed in the region of tissue damage. To justify association between byproducts of oxidative damage and seizure, it would be particularly important to undertake antioxidant intervention studies, based on inhibiting oxidative damage and assessment of the impact on both type and frequency of seizures.

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